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A suggestion for royal jelly specifications

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This article proposes guidelines for quality standards of royal jelly. The proposals are based on two sets of data; the first from our study of the factors that may affect the royal jelly's chemical composition (protein and sugar supplementation of beehives) and the second on the analysis of a great number of samples from across Greece to establish natural variability of this product. We compared our findings with the adopted national limits, the proposals of the working group of the International Honey Commission (IHC), and the draft proposal of the International Organization of Standardization (ISO). The studied parameters included moisture, total proteins, sugars (fructose, glucose, sucrose, total sugars), and 10-hydroxy-2-decenoic acid (10-HDA). Our results indicate that the limits for royal jelly in some countries should be amended and the proposals of the IHC and the ISO reviewed in view of recent data on variability. We believe that our proposals could be considered for setting global standards for royal jelly, as they incorporate national legislations, proposals of scientific groups, experimental data, and updated information.

KEY WORDS: *10-HDA; fructose; glucose; Greece; international guidelines; IHC; ISO; moisture; national standards; sucrose; sugar; total proteins*

Royal jelly (RJ) is a creamy, whitish, strongly acidic secretion from the mandibular and hypopharyngeal (cephalic) glands of nurse bees (*Apis mellifera*). It can change an ordinary worker bee larva, which usually lives a month and a half, into a large queen bee that can live up to five years and easily lay her own weight in eggs each day. The pharmaceutical properties of RJ for humans have been extensively studied and several claims were made regarding its beneficial effects on the immune system, metabolism, vascular and glandular function, skin health, heart function, and cholesterol and lipid control (1-4). However, these claims have been contested by the European Food Safety Authority, which concluded that no relationship has been evidenced between the consumption of royal jelly and the claimed effects (5).

In spite of this unfavourable opinion, RJ is still one of the most popular beehive products in many countries. Official data on the production of RJ are not available, but it is estimated that the world production is a few thousand tonnes per year (6). China alone is estimated to produce about 2,000 tonnes per year, or about 60.0 % of the global production (7). RJ is also produced in Korea, Taiwan, Japan, Mexico, and the EU countries such as Spain, Greece, France, and Italy.

Although RJ is a promising product with added financial value for the beekeepers, the industry is growing very slowly, and the local demand in many countries is covered by import from China. What largely discourages beekeepers from expanding their businesses is the lack of quality criteria and control of authenticity and geographical origin.

Today, there are no standards at the European or International level for bee products other than honey, but several countries have established national standards or guidelines. The first country that set the criteria for RJ was Argentina in 1979 (8), followed by Bulgaria in 1984 (9), Poland in 1996 (10), Turkey in 2000 (11), Brazil in 2001 (12), Serbia in 2003 (13), Switzerland in 2005 (amended in 2014) (14, 15), Japan (16) and China (17) in 2008, India in 2012 (18), and Korea in 2014 (19). A few years ago, a working group of the International Honey Commission (IHC) prepared a preliminary proposal for the standardisation of RJ based on information they collected (7). Recently, the International Organization for Standardization (ISO) issued a draft of international standards (20) which distinguishes RJ produced by bees fed with natural food (type 1) and RJ produced by bees fed with other nutrients such as proteins and carbohydrates (type 2).

Greece has no quality standards for RJ, but the laboratory of Apiculture of the Aristotle University has been commissioned by Hellenic Ministry of Rural Development and Food to draft national standards. For this purpose we first performed experiments to find factors that may affect chemical synthesis of RJ and then we collected a great number of samples from different areas of Greece and analysed them to establish natural variability in the physicochemical parameters of the product. To suggest standards for RJ, we compared our data with current

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legislations of a number of countries, the proposal of the IHC working group, and the ISO draft. Considering that we gathered national legislations, proposals of scientific groups, experimental data, published information, and beekeepers opinions, our findings might serve well for defining standards for RJ all over the world.

MATERIALS AND METHODS

Sampling from feeding experiment

We collected samples of RJ from feeding experiments with bee colonies from the apiary of Aristotle University of Thessaloniki, Greece. The experiment involved syrup and protein supplementation.

The effect of protein supplementation on RJ composition was studied in fifteen colonies which were divided into five experimental groups: group A was fed with a commercial product of unknown protein composition, group B with soybean flour (mixed with 10 % of honey), group C with smashed pollen collected with pollen traps in our apiary, group D with sugar-based candy of our own making that contained 10 % of pollen, and group E received no supplements during RJ production (control). Four consecutive graftings of 30 queen cells each were done per colony. All experimental colonies were provided by pollen traps to exclude the incoming pollen. In addition, combs with stored pollen (bee-bread) were removed before starting the experiments.

The effect of carbohydrate supplementation on RJ composition was studied in six queenless colonies. The experiment started with grafting artificial cells without syrup, three graftings per colony. After that, the colonies got back again the queen cells to produce RJ, but this time they were provided with 1.0 L of sugar syrup (1:1) at the day of grafting. This way the same six colonies participated in consecutive grafting with or without feeding. The procedure was repeated three times to give 54 samples from the colonies that produced RJ without supplementation and other 54 samples from the colonies fed with syrup. Between each repetition the colonies were left unfed for a period of 20 days.

Sampling from RJ producers

The collection of RJ samples from producers was based on the following protocol: RJ producers declared the time of collection, the area, whether the colony was fed or not, and the number of colonies involved in RJ production. The origin of the samples was verified by analysing the pollen spectrum and comparing the bee flora of each area. We collected 176 samples from 34 major beekeeping areas of Greece.

Chemical analysis

Water content was determined using the drying oven method (21).

To determine10-hydroxy-2-decenoic acid(10-HDA) we used high-performance liquid chromatography with photodiode array detection (HPLC-DAD) (Agilent Technologies 1200, Tokyo, Japan). Samples of 0.2 g of RJ were mixed with 1.0 mL of pure water produced using a Simplicity 185 system (Millipore, Molsheim, France), 0.6 mL of HCl (1 mol mL^{-1}) (Chem-Lab, Zedelgem, Belgium), and 0.4 mL of methyl-4-hydroxybenzoate (1.0 mg mL^{-1}) (Sigma-Aldrich, St. Louis, MO, USA) as internal standard. The volume was filled up to 10.0 mL with HPLC purity ethanol (Merck, Darmstadt, Germany). The mixture was placed in the ultrasonic water bath for 10 min and then filtered through a 0.22-μm disposable syringe filter (Millipore, Darmstadt, Germany) before the injection..

For the separation we used an Athena C_{18}-WP column (3 μm x 150 mm x 4.6 mm) at the temperature of 30 °C. The mobile phase was a mixture of methanol (Chem-Lab, Zedelgem, Belgium), pure water, and orthophosphoric acid (Chem-Lab, Zedelgem, Belgium) (50:50:0.3, v/v/v). The injection volume, flow rate, and the detector's wavelength were 10 μL, 1.0 mL $min^{-1}$and 210 nm, respectively.

For the quantitation of the carbohydrate content (fructose, glucose, and sucrose) in RJ we used HPLC with a refractive index detector (RID) (Agilent Technologies 1200, Tokyo, Japan). A sample of 0.5 g of RJ was mixed with the reagents $C_6FeK_4N_6$ x $3H_2O$ (Sigma-Aldrich) and $(CH_3COO)_2Zn$ x $2H_2O$ (Merck) and transferred to volumetric flasks. The flasks were filled with 5 mL of a methanol/water solution (25:75, v/v). Before injection, the mixture was filtered through a disposable syringe filter. The sugars were separated on a Zorbax Carbohydrate Analysis Column (4.6 mm x 150 mm x 5 μm) (Agilent Technologies 1200)using a mixture of acetonitrile (Sigma-Aldrich) and water (80:20, v/v) as mobile phase at a flow rate of 1.3 mL min^{-1}. The injection volume was 10 μL.

To determine total nitrogen content we used the Kjeldahl method following the AOAC procedure (22). A sample of 0.5 g of RJ was mixed with concentrated sulphuric acid (Merck) and placed in a digestion tube with the presence of a catalyst (Thompson & Capper Ltd, Cheshire, UK) which helps to convert amine nitrogen to ammonium ions. The digestion tube was heated until the mixture became light blue. The sample was then cooled, and the tube placed in a Kjeflex K-360 steam distillation unit (Buchi, Flawil, Switzerland). During distillation, the ammonium ions were converted into ammonia gas in the presence of NaOH (30.0 %, w/v) (Chem-Lab, Zedelgem, Belgium), which in turn was transferred into a trapping solution of H_3BO_3 (4.0 %, w/v) (Merck), dissolved, and converted back to ammonium ion. For titration we used a 0.1 mol L^{-1} HCl solution added with a MettlerTolledoT50 titrator (Schweiz, Switzerland). Finally, we estimated the crude protein content (%, w/w) using the factor of 6.25.

Statistical analysis

Total protein and sugar content were compared between the groups with one-way analysis of variance (ANOVA) using the IBM SPSS statistics software (ver. 19) (New York, NY, USA), and the significance level of the test was set at 0.05 (α=0.05).

Probability plots were used to compare the analysed data against the proposed or established limits from different countries.

RESULTS AND DISCUSSION

Quality standards of RJ

Table 1 shows our findings in Greek samples, current national limits, IHC (7), and the ISO draft (20) proposals and reports from other studies.

Moisture

In 96.7 % of the 176 Greek RJ samples the water content ranged between 60.0 and 70.0 % (Figure 1). As this range coincides with the limits proposed by the IHC and adopted by Brazil and Argentina (Serbia, Poland, and Switzerland set only the upper limit) (Table 1), we also propose it as an international standard. We believe that this range reflects the natural variation for this parameter.

The current national limits of a number of countries (see Table 1) are based on a very narrow moisture range that currently excludes 30 % of the samples from our study, mainly because the research on which this narrow range is based probably used a very small number of samples. Moisture content is also affected by delays in harvesting. Most producers harvest the product three days after the grafting of one-day-old larvae. Our research has shown that delayed collection (forth day) results in very low water content (<50 %), because bees suck part of RJ water. Similarly, early collection of RJ (on the first day after grafting) also results in a product with low water content (<60 %) (23).

10-HDA

The lipid 10-HDA is one of the most crucial components of RJ, to which the principal biological activities of the product are attributed. The values of 10-HDA in 97 samples that we analysed ranged from 0.8 to 6.5 % (Figure 2). The limit (>1.4 %) proposed by ISO and IHC, which conforms with the national directives of Turkey, Japan, Switzerland, India, China, and Korea makes 18.0 % of the Greek samples non-compliant, while the Brazilian limit of >2.0 % excludes 48.0 % of the Greek samples. The limit of 1.4 % should be reconsidered, as several studies indicate that 10-HDA may be present in authentic RJ samples in lower concentrations (25-27). We therefore suggest 1.0 % as the lower limit, which covers 99.0 % of our samples. We also recommend an upper limit of 6.0 % to restrain adulteration from adding synthetic 10-HDA, which is widely available in international trade.

Proteins

Feeding experiments indicated that protein supplements did not significantly affect the protein content of RJ

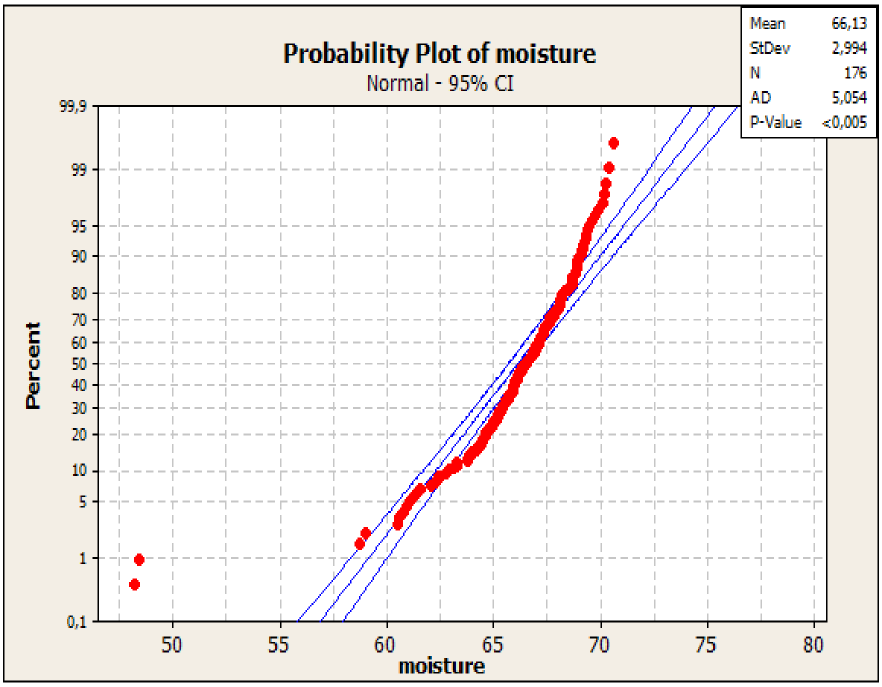

Figure 1 *Moisture content in 176 samples of royal jelly collected by Greek producers across the country*

Table 1 *Comparison between the results of this study, national standards, and published information regarding royal jelly (RJ) composition*

Parameter	Greek RJ	National limits and proposals		Values from international bibliography
Moisture %	Mean: 66.1 min-max: 46.8-73.2 Std:3.0 Proposed limits:60-70 % n=176	ISO Japan Turkey India Bulgaria China Korea Poland IHC Brazil Argentina Serbia Switzerland	62.5-68.5 62.5-68.5 62.5-68.5 62.5-68.5 62.5-67.0 62.5-67.5 65.6-68.5 max: 67.0 60-70.0 60-70.0 60-70.0 max: 70 max: 70	65.3; n=1 (33) 53.8-66.6; n=27 (34) 63.7; n=8 (35) 60.2-65.2; n=5 (36) 59.1-65.8; n=13 (24) 58.8 - 65.8; n=69 (30) 61.4-67.6; n=7 (32) 67.8-69.4; n=3 (37)
10-HDA %	Mean: 2.32 min-max: 0.8-6.5 Std:1.0 Proposed limits: 1-6 % n=97	ISO IHC Turkey Switzerland Japan Brazil Korea India China	min: 1.4 min: 1.4 min: 1.4 min: 1.4 min: 1.4 min: 2.0 min: 1.4 min: 1.4 min: 1.4	1.26-2.25; n=20 (38) 0.75-2.54; n=10 (25) 1.58-3.39; n=7 (32) 0.75-2.54; n=7 (26) 0.8-3.2 (27)
Protein %	Mean: 13.6 min-max: 10.5-19.6 Std:1.9 Proposed limits: min 10 % n=176	Brazil China Turkey Korea India Argentina Japan Bulgaria Poland SO IHC	min: 10 min: 11 11.0-14.5 11.0-14.5 11.0-15.0 11.0-15.0 12.0-15.5 12.0-17.0 13.5 -20.0 11.0-18.0 9.0-18.0	13.4; n=1 (33) 15.8-16.7; n=3 (37) 12.0-14.0; n=7 (32) 12.2-19.6; n=13 (24) 9.5-19.6; n=69 (30)
Fructose %	Mean: 3.98 min-max: 2.1-7.3 Std:1.0 Proposed limits 2-8 % n=151	ISO IHC	2.0-9.0 3.0-13.0	5.5; n=1 (39) 0.5-6.3; n=3 (37) 3.64-5.47; n=13 (24) 2.7-7.7; n=69 (30) 2.3-6.9; n=97 (31) 2.3-7.3; n=290 (28)
Glucose %	Mean: 4.2 min-max: 2.4-6.9 Std: 0.9 Proposed limits: 2-8 % n=151	ISO IHC Brazil	2.0-9.0 4.0-8.0 max: 10.0	6.1; n=1 (39) n.d.-2.7; n=3 (37) 2.01-5.87; n=13 (24) 2.01-8.68; n=69 (30) 3.7-8.2; n=97 (31) 2.9-8.0; n=290 (28)
Sucrose %	Mean: 2.86 min-max: 0.0-9.3 Std:2.0 Proposed limits: type 1: max 3 % type 2: max 6 % n=151	ISO IHC Brazil Bulgaria	max: 3.0 (type 1) max: 6.0 (type 2) 0.5-2 max: 5.0 max: 4.5	1.45; n=1 (39) n.d.-3.0; n=3 (37) 0.36-3.59; n=13 (24) 0.04-5.08; n=69 (30) n.d.-2.1; n=97 (31) n.d.-1.7; n=290 (28)* 0.1-5.1; n=27 (28)**
Total sugars %	Mean: 11.03 min-max: 7.2-16.7 Std:2.0 Proposed limits 7-17 % n=151	ISO IHC Poland Bulgaria	7.0-18.0 7.0-18.0 6.5-18.0 9.0-13.0	10.45; n=1 (33) 15.3-21.5; n=7 (32) 13.8; n=1 (39) 11.4-11.5; n=3 (37) 8.05-13.5; n=13 (24) 8.05-16.61; n=69 (30) 6.9-16.0; n=97 (31) 8.1-16.8; n=290 (28)* 10.7-17.0; n=27 (28)**

**Samples collected in bees not fed with syrup*
***Samples collected in bees fed with syrup*

(*P*=0.159; Table 2). The minimum levels all exceeded the 9.0 % lower limit proposed by the IHC working group (10), and the highest level measured in our university apiary samples (group B,13.8 %) is far below the proposed upper limit of 18.0 %.

The range of protein content in the 176 samples collected from Greek producers was 10.5-21.0 %, and the average was 13.6 % (Figure 3).

The IHC, ISO, and national limits show great variation (Table 1), possibly due to different methods of analysis or to the small number of samples used in the studies on which they were based. The differences between the national ranges may impede trade between the countries and even within the same country, because, according to our findings, they fail to reflect authentic values of RJ. It turns out that somewhere between 20 % and 70 % of our samples may not match current national criteria.

We therefore propose that only the lower protein limit of >10.0 % is adopted for all countries, since our feeding experiments with different protein diets did not show any significant effect on protein concentration in royal jelly. This limit has also been set by Brazil.

Sugars

Our feeding experiments have shown that the fructose content in RJ decreases about 1.0-2.0 % in colonies fed with syrup (Table 3). A similar decrease was found by Daniele and Casabianca (29). Although this effect is statistically significant, the fructose content falls within the limits of samples collected from unfed colonies. The mean fructose content in the151 samples from Greek producers was a bit lower than in our experiments (3.98 %) and ranged between 2.1 % and 7.3 % (Figure 4), if we exclude one outlier sample with 9.8 %, probably due to natural variability.

Table 1 indicates that no national limits have been established for fructose in RJ so far, but ISO proposes the limit of 2.0-9.0 % for naturally fed bees (RJ type 1) and the IHC working group 3.0-13.0 % (7). The latter range is quite exclusive with the lower limit (20 % of our samples do not meet it) and at the same time allows an unreasonably high upper limit, considering that hardly any study reports fructose levels higher than 8.0 % (16, 19-21). In contrast, the ISO limits seem to encompass most of the reported ranges and greatly overlap with our proposal (2.0-8.0 %).

Similar to fructose, glucose was also significantly lower in bees fed on syrup (0.5-1.0 %, Table 3), but again this decrease falls within the limits of unfed colonies. Our samples from Greek producers ranged between 2.4 % and 6.9 % (Figure 5), except for one outlier with extremely low glucose (0.9 %).

The range proposed by ISO for RJ type 1 is 2.0-9.0 %. Our experimental and producers' samples fall comfortably within this range, but 40.0 % fail to meet the lower limit of 4.0 % proposed by the IHC working group, which is extremely high and does not correspond to our findings or recent publications (see Table 1). Unlike ISO and IHC, Brazil has set only the upper limit of 10.0 % and is the only country defining this standard.

We propose the range of 2.0-8.0 % for glucose in RJ, as it encompasses all of our samples (except for one outlier) and most of the reported ranges in studies across the world (24, 27, 29, 30).

As expected, sucrose levels in our feeding experiment were significantly higher in bees fed with syrup than in unfed bees (Table 3). These findings have also been supported by Daniele and Casabianca (28).

In RJ samples obtained from Greek producers the average sucrose level was 2.9 %, but the range was quite wide (0.0-9.3 %) (Figure 6). Considering the sucrose

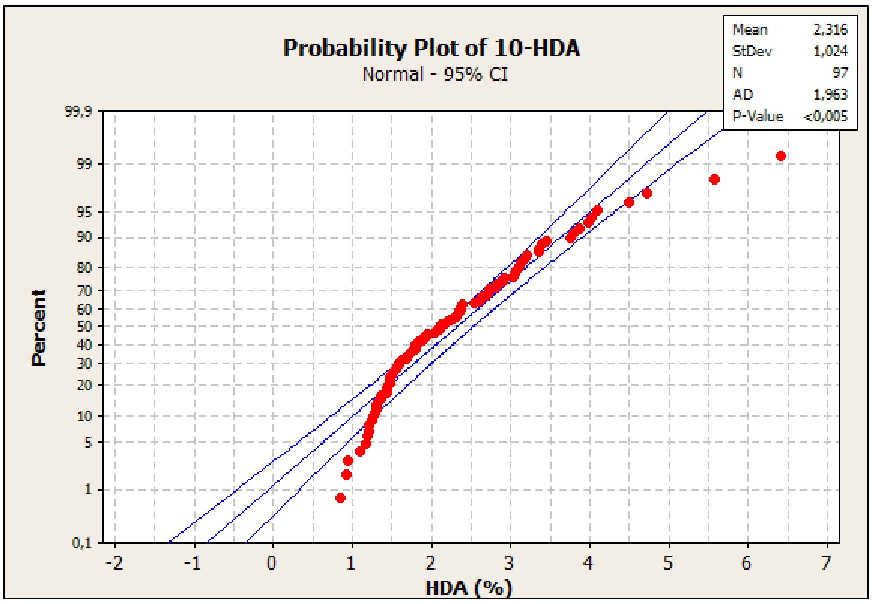

Figure 2 *10-HDA in 97 samples of royal jelly collected by Greek producers across the country*

Table 2 *Protein content in samples of royal jelly produced from colonies fed with protein supplements (feeding experiment)*

	% Protein Content	
	Mean	**min-max**
Group A (Commercial product)	11.8 (±0.9)	10.7 - 13.0
Group B (Soybean flour + 10 % honey)	12.7 (±0.7)	12.0 - 13.8
Group C (Smashed pollen)	12.3 (±1.0)	11.0 - 13.7
Group D (Candy + 10 % pollen)	12.4 (±0.7)	11.4 - 13.6
Group E (Control – no feeding)	12.1 (±0.8)	11.1 - 13.1

The results are presented as mean±standard deviation. There was no significant difference ($p<0.05$*) between the groups. All data were evaluated using Duncan's Multiple Range test*

maximum of 1.6 % in the unfed bees in our feeding experiment, this finding suggests that many Greek beekeepers feed their bees with syrup during RJ production, as they believe that feeding with syrup stimulates the bees to produce more RJ.

Brazil has adopted 5.0 % as the upper limit, Bulgaria 4.5 %. ISO has proposed 3.0 % for RJ produced by unfed bees. If we apply this limit to the samples collected by Greek producers, 40 % of them do not meet this criterion. With the IHC range of 0.5-2.0 % the exclusion soars. Furthermore, the IHC lower limit does not seem to rest on actual findings from unfed colonies.

In any case, RJ producers should adjust their colony feeding practices. Published information shows that sucrose content from unfed colonies is less than 3.0 % and from fed colonies less than 6.0 % (27, 28). Therefore, we support the proposal by ISO for RJ type 1 and type 2.

Table 1 shows that the range of total sugars (fructose, glucose, and sucrose) in most of the 151 samples from the Greek producers falls within the proposed national and ISO/IHC ranges. We propose a slightly stricter range than ISO and IHC, 7.0-17.0 %, which covers 100 % of our producer sample (Figure 7) and is in agreement with the Daniele and Casabianca research (28) on a great number of samples.

CONCLUSIONS

The number of samples, the methods of analysis, and beekeeping manipulations that may influence the chemical composition of RJ are very important in evaluating RJ parameters. Different limits that had been adopted by some countries are not always applicable and may hinder commerce between countries. The standards we propose in this study are an attempt to overcome current limitations imposed by inconsistent standards, as they encompass our experimental data, current national legislations, proposals of scientific groups, published bibliography, and beekeepers' opinions. We hope this article will open a fruitful discussion that might lead to adopting universal standards for royal jelly.

Acknowledgements

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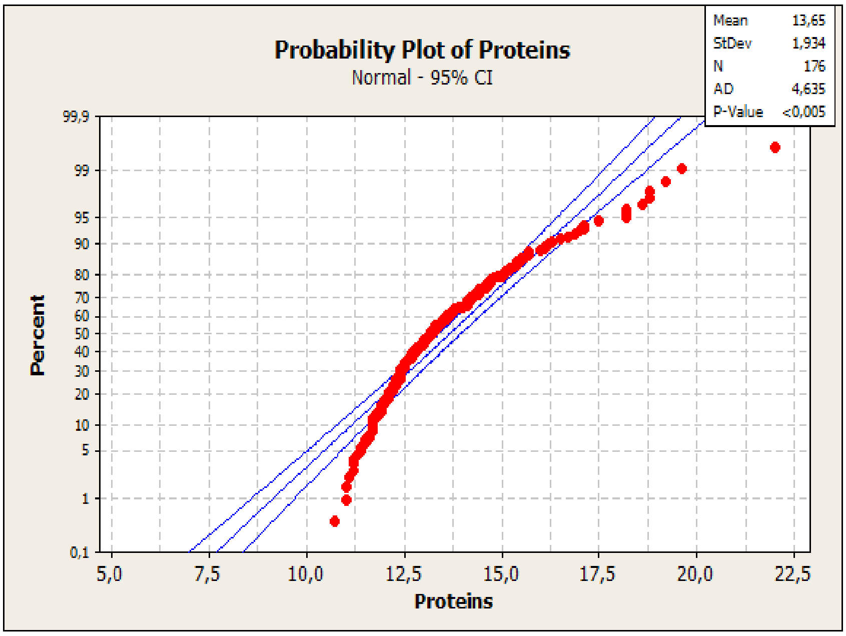

Figure 3 *Total proteins in 176 samples of royal jelly collected by Greek producers across the country*

Table 3 *Mean content of the main sugars in samples of royal jelly with and without feeding (n=54)*

Sugars	Without Feeding		With syrup feeding	
	Mean	Min - Max	Mean	Min - Max
Fructose %	4.3[a] (±0.81)	2.9 - 6.3	3.5[b] (±0.70)	2.3 - 5.0
Glucose %	4.6[a] (±0.99)	3.0 - 6.8	3.7[b] (±0.77)	2.6 - 5.9
Sucrose %	0.6[a] (±0.49)	0.0 - 1.6	4.6[b] (±1.57)	2.5 - 7.8

Values with different superscript letter (a>b) within the row indicate significant differences ($p<0.05$*). All data were evaluated using the t-test.* $p=0.003$ *for fructose;* $p=0.008$ *for glucose; and* $p=0.001$ *for sucrose*

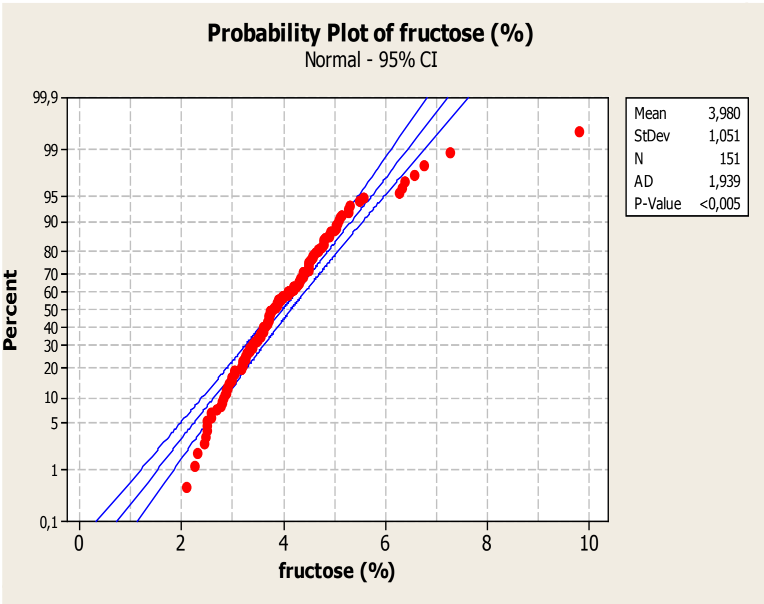

Figure 4 *Fructose in 151 samples of royal jelly collected by Greek producers across the country*

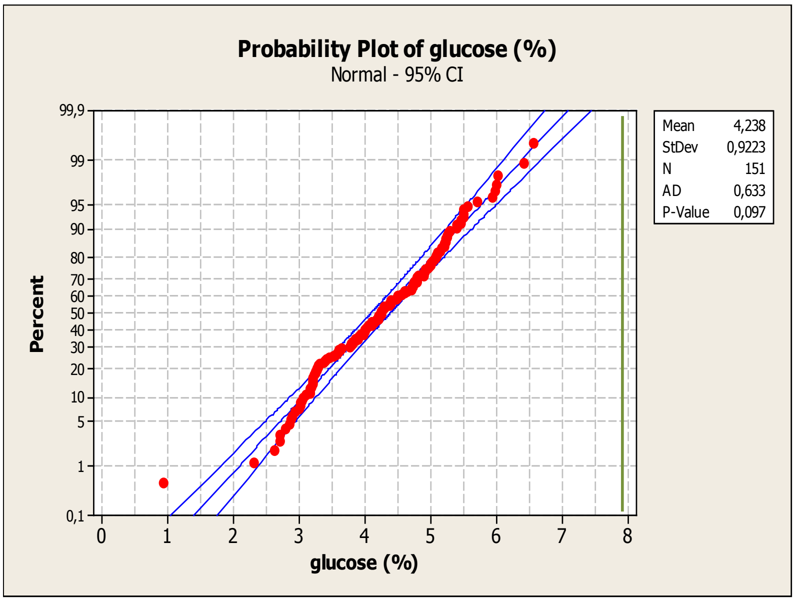

Figure 5 *Glucose in 151 samples of royal jelly collected by Greek producers across the country*

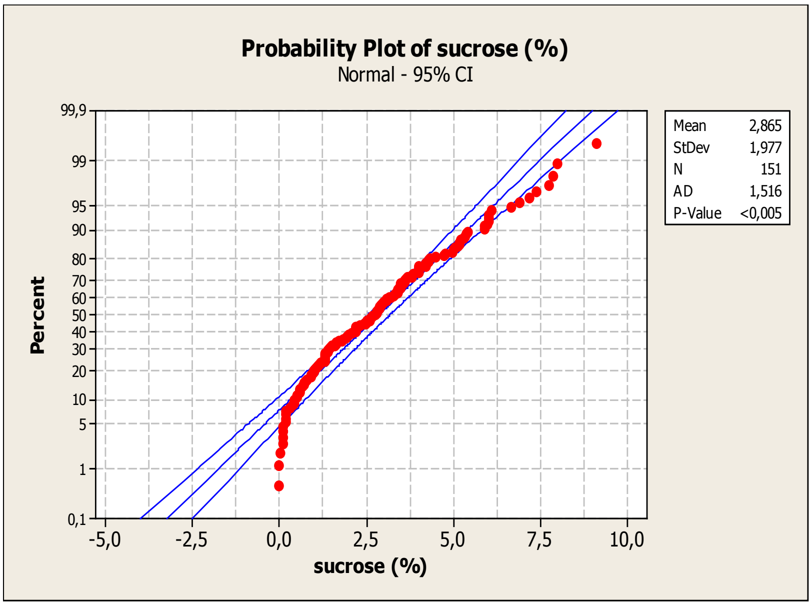

Figure 6 *Sucrose in 151 samples of royal jelly collected by Greek producers across the country*

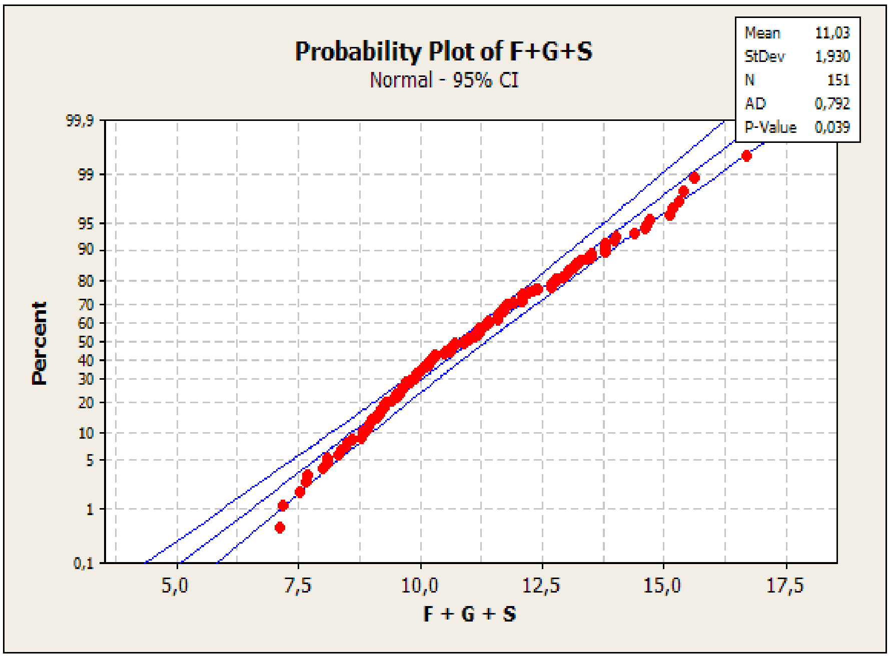

Figure 7 *Total sugars in 151 samples of royal jelly collected by Greek producers across the country*

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Prijedlog globalnih standarda za matičnu mliječ

U ovome se članku predlažu međunarodne smjernice za kakvoću matične mliječi. U prvom dijelu istraživanja analizirali smo koliko dohrana bjelančevinama i šećerima utječe na kemijski sastav matične mliječi, a u drugom smo analizirali velik broj uzoraka matične mliječi prikupljene iz cijele Grčke kako bismo ustanovili prirodnu varijabilnost ovog proizvoda. Svoje smo rezultate usporedili s usvojenim standardima različitih država, prijedlogom standarda Međunarodne komisije za med (International Honey Commission - IHC) te nacrtom prijedloga Međunarodne organizacije za normizaciju (International Organization of Standardization - ISO). Analizom su obuhvaćeni sljedeći parametri: vlažnost, ukupne bjelančevine, šećeri (fruktoza, glukoza, saharoza) te 10-hidroksi-2-decenska kiselina (10-HDA). Naši rezultati upućuju na to da neke zemlje trebaju izmijeniti i dopuniti svoje standarde, a IHC i ISO trebaju ponovo razmotriti svoje prijedloge u svjetlu najnovijih podataka o prirodnoj varijabilnosti matične mliječi. Smatramo da bi naš prijedlog mogao poslužiti za utvrđivanje međunarodnih standarda za matičnu mliječ s obzirom na to da obuhvaća podatke iz zakona i propisa pojedinih država, prijedloge znanstvenih i stručnih skupina, podatke iz istraživanja te najnovije informacije iz literature.

KLJUČNE RIJEČI: *10-HDA; bjelančevine; fruktoza; glukoza; Grčka; IHC; ISO; međunarodne smjernice; nacionalni standardi; saharoza; šećer; vlažnost*